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The significance of the analysis of noncholesterol sterols and profile of fatty acids in various pathophysiological states

Ph.D. thesis summary

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CONTENT

1 INTRODUCTION

The compound similar to cholesterol was first mentioned as early as two hundred forty years ago (Poulletier de la Salle 1769), but present term "cholesterol" should be inscribed to another French, M.E. Chevreul (1815)[1]. Since that time, thousands of steroids, compounds with steran tetracyclic system, have been isolated in living organisms. The vast structural diversity of steroids is reflected in broad spectrum of their effects. The subtle part of steroids, sterols, is characterized with the hydroxyl group on the third carbon atom of the steroid core. This group is mainly represented by cholesterol and its precursors as well as by plant sterols (phytosterols). The intermediates of cholesterol biosynthesis and phytosterols could be named together as noncholesterol sterols. Until recently, it was assumed that cholesterol precursors do not possess any clinically important features and that the variety of precursors is simply due to complicated final structure of cholesterol. Nowadays, some of these intermediates are supposed to play a role. Together with the obvious fact that the concentration of intermediates reflects the level of biosynthesis of cholesterol, we have another impetus for the analyses of these compounds.

Fatty acids are another large group of lipids with no less voluminous variety of effects. They are present in biological membranes and function as ligands of nuclear factors, signal molecules and precursors of several types of compounds, with eicosanoids on one end and miscellaneous functional derivatives on the other. The essentiality of fatty acids with two and more double bonds adds another nutritional aspect into the rich mosaic of effects of fatty acids on our organism. This group of fatty acids is life important and therefore, it should deserve more attention even of lay public.

The structural motif of sterol and fatty acid chain cross-talk on several levels of metabolism. The cholesterol and fatty acid molecules interact in cell membranes and they are responsible for physical properties of biological membranes. Cholesterol and fatty acid form the molecule of cholesteryl ester, the storage form of cholesterol in the intracellular space and inner part of lipoprotein particles in extracellular compartment (mainly plasma). Furthermore, sterol as well as fatty acid structures are important structural motifs of ligands, which take part in transcriptional mechanisms governing many of the genes of the lipid homeostasis. The target genes are in several cases regulated by both cholesterol and fatty acids.

The cohesion of the metabolism of cholesterol and fatty acids, which reaches from physical interaction in the membrane as far as to the competition as ligands for gene transcription, raises the question whether there is connection in the metabolism of cholesterol

and fatty acids, especially in the case of pathophysiological events with disturbed lipid metabolism (cholesterol homeostasis and transformation of fatty acids).

2 AIM OF THE THESIS

The aims of the Thesis were as follows:

1. To modify the methodological approach for the determination of noncholesterol sterols and fatty acids in human plasma by the capillary gas chromatography.

2. With the respect of data obtained by the analysis of noncholesterol sterols and fatty acids as well as other biochemical analyses, to suggest (in chosen diseases) possible pathological mechanisms derived from the relationship of metabolism of cholesterol and fatty acids.

3 METHODS AND PATIENTS

Analytical Methods

The analyses of the fatty acid profile in lipid classes of plasma (cholesteryl esters, triacylglycerols and phospholipids, phosphatidylcholines, resp.) and triacylglycerols of adipose tissue were carried by capillary gas chromatography. The concentrations of plasma noncholesterol sterols were also analysed by the same method. The supplemental biochemical analyses were performed with standard methods in lipid or central laboratory of General teaching hospital; if the separation of LDL was necessary, the sequential ultracentrifugation step[2] was introduced into the sample handling scheme. The extraction of lipids from brain tissue followed the procedure by Folch[3] and the separate lipid classes in the extract were quantitatively analysed by thin-layer chromatography with flame-ionisation detection[4].

Experimental Model

Male Wistar rat were chosen for the experimental study; prior to the study launch, they were grouped according to the plasma triacylglycerol and cholesterol levels so the groups did not differ from each other. The rats were caged by four in standardized conditions and they were administered with hypolipidemic drugs or placebo.

Patients

The groups of patients under investigation included persons with anorexia nervosa, proteino-energetic malnutrition, the obese, hyperlipidemic patients as well as the control group. The anthropometrical and clinical characteristics of all the persons were acquired by medical examination. The control group included the employees of the IVth Department of Medicine, 1 LF UK, and their relatives, or person enrolled on another voluntary basis. For the inclusion or exclusion of the person into the relevant group, we applied appropriate criteria [5,6,7]. All the participants were informed about the study and signed informed consent with the study. All studies were approved by ethical committees.

Statistical Evaluation of the Data

Data from the studies were statistically processed with commercial software STATISTICA® for Windows; during the procedure, both parametrical and nonparametric one-dimensional approach was used. Multidimensional analysis of the data was used for multiple distribution comparison with Hotteling t-test. Correlation analysis took the advantage of the same program with the exception of comparison of regression lines; in this case, the program in the Microsoft Excel® for Windows environment was configured on the basis of linear regression theory[8].

4 PRINCIPAL RESULTS OF THE THESIS

4.1 Methodological Studies

Effect of Column and Software on Gas Chromatographic Determination of Fatty Acids – Study I

Four capillary columns (A: CP-WAX 52 CB 25 m x 0.25 mm; B: CP WAX 52 CB 30 m x 0.25 mm; C: CP-WAX 58 CB 25 m x 0.25 mm, Chrompack; D: OMEGAWAX™ 320 30 m x 0.32 mm, Supelco) and two integration software (Mosaic® v.5.10, Chrompack and CSW v.1.7, Data Apex®) were compared for analysis of fatty acids. Column A was mounted stepwise in two different instruments. Fatty acids of blood plasma phosphatidylcholine and standard mixture of saturated fatty acids were analysed as methyl esters under identical chromatographic conditions. Both integrating software did not differ significantly in most results; differences were observed only for minor components: 16:1n-9, 20:0 and 20:2n-6. Increased values for 16:1n-9 and 20:2n-6 integrated by MOSAIC are caused by cointegration of two poorly resolved peaks: fatty acid and impurity from sample matrix. Lower values for 20:0 are caused by incomplete integration of minor peak. Differences between columns were observed mostly for minor fatty acids. Linear calibration dependences measured with standard mixture of saturated fatty acids (carbon number 10–24) were observed in wide range of concentrations (three orders). Slope close to unity and minimal value of intercept confirmed theoretical relations when analyses are run under optimal conditions.

Effect of analytical matrix on fatty acid analysis – Study II

Profiles of fatty acids in lipid classes of plasma and ultracentrifugally separated lipoproteins were analyzed by capillary gas chromatography. Contribution of individual steps of the whole procedure was calculated on the basis of the multiple analyses. GC contributes to the overall error by the smallest part. Contribution of the extraction, thin-layer chromatography separation and methylation processes might be comparable. The value of relative standard deviation is indirectly proportional to the amount of analyzed component. Comparison of FA profiles in lipid classes of very low density lipoproteins and low density lipoproteins revealed highly individual differences. Increased content of arachidonic and docosahexaenoic acids in triglycerides of low density lipoproteins are in agreement with formerly published results.

Comparison of Fatty Acid Profile in Serum Phospholipids and Phosphatidylcholines – Study III

The aim if this study was to statistically evaluate the effect of individual steps of the fatty acid analysis (thin-layer chromatography, transmethylation reaction and gas chromatography) in the isolated phosphatidylcholines and the fatty acid profile compare with that of phospholipids.

Both types of analyses revealed significant differences for most fatty acids. High level of significances for the differences between fatty acid profile in phosphatidylcholines and phospholipids were due to repeated analyses with little total error of the method. However, these differences were negligible compared to deviation within the biological samples and they did not exhibit systematic bias for the samples analysed.

Analyses of Noncholesterol Sterols – Study IV

Usually, clinical practice requires the analysis of the sum of free and esterified sterol molecules from the unsaponifiable part of the serum/plasma. The optimization of the analytical method was focused on some steps of the analytical process.

Acetylation was preferred to analysis of free sterol due to better analytical conditions. The acetylation procedure gave the highest reproducibility (RSD 2 %) and almost the highest yield at room temperature for 24 hours in the dark. Heating of the samples is not recommended. Injection mode for further analyses was set to split because it gave higher slopes in calibration dependencies; moreover, in some clinical samples, in the splitless mode some “ghost” peaks appeared. For the injector configuration in use, we did not prove the different effect of injection with 7 cm and 5 cm needle.

The response factors for different injection volumes in case of 5α-cholestanol, lathosterol and β-sitosterol are comparable. With the injection volumes reaching minimal values, the more pronounced effect of matrix giving poorer reproducibility should be expected. The possibility of analysis of both cholesterol and noncholesterol sterols in one chromatographic run was studied. It was not possible to obtain reproducible results for one run analysis, therefore this limitation was overcome with two dilution and two internal standard method, i.e. two chromatographic analyses are necessary.

Furthermore, the contribution of separate steps of the plasma noncholesterol analysis was determined. The quantitative assessment of lanosterol and 5α-cholestanol is biased with error excluding the use of this analytical method for clinical samples.

4.2 Experimental Study

Hypolipidemic Drugs Can Change the Composition of Rat Brain Lipids – Study V

Hypolipidemic drugs are potent serum cholesterol lowering agents used for prevention of coronary heart disease. In addition to their cholesterol lowering effect, the hypolipidemic drugs exhibit both pleiotropic beneficial and various neurological side effects. Therefore, we analysed effect of these drugs (three statins, reversible competitive inhibitors of cholesterol biosynthesis and fenofibrate, the drug affecting primarily plasma high triacylglycerol with indirect effect on plasma cholesterol) on membrane lipid composition in the rat brain tissue. Male Wistar rats were given 0.1 mg of fenofibrate, lovastatin, pravastatin, fluvastatin or placebo once daily for six weeks. In lovastatin and pravastatin treated groups, decreased cholesterol and increased ceramide monohexoside contents in the brain tissue were observed in comparison with control group. Fluvastatin and lovastatin treatment resulted in increased sphingomyelin and decreased diphosphatidylglycerol contents. The most important changes in fatty acid profile were observed in ceramide monohexosides: in fluvastatin group, decreased content of saturated and increased content of polyunsaturated fatty acids. Fenofibrate dosage led to decreased content of saturated fatty acids in phosphatidylethanolamines.

4.3 Clinical Studies

Composition of Plasma Fatty Acids and Non-Cholesterol Sterols in Anorexia Nervosa – Study VI

Anorexia nervosa is a model of simple starvation accompanied by secondary hyperlipoproteinemia. Pattern of plasma fatty acid influences levels of plasma lipids and lipoproteins. Concentration of plasma lathosterol is surrogate marker of cholesterol synthesis *de novo*, concentrations of campesterol and β-sitosterol reflect resorption of exogenous cholesterol. The aim of the study was to evaluate fatty acids in plasma lipid classes and their relationships to plasma lipids, lipoproteins, cholesterol precursors and plant sterols.

We examined 16 women with anorexia nervosa and 25 healthy ones. Patients with anorexia nervosa revealed increased concentrations of total cholesterol, triglycerides, HDL-cholesterol, campesterol and β-sitosterol. Moreover, decreased content of n-6 polyunsaturated fatty acids in all lipid classes was found. These changes were compensated by the increased content of monounsaturated fatty acids in cholesteryl esters, saturated fatty acids in triglycerides and both monounsaturated and saturated fatty acids in phosphatidylcholine. The most consistent findings in fatty acid pattern were decreased content of linoleic acid (LA, 18:2n-6) and raised content of palmitoleic acid (POA, 16:1n-7) in all lipid classes.

Plasma Fatty Acids and Sterols in Protein-energetic Malnutrition – Study VII

Proteino-energetic malnutrition, as a result of different types of starvation, represents serious complication for patients in critical care. The aim of our study was to find changes in lipid and lipoprotein parameters in proteino-energetic malnutrition, which usually are not included in standard profile of analyses. Group of patients with PEM consisted of 22 persons (12 M/10 F), the control one of 35 healthy persons (10 M/25 F).

Patients with proteino-energetic malnutrition had lower concentrations of total cholesterol both in plasma and lipoprotein particles as well as apoA-I compared with controls. The lathosterol concentration was not changed, but lathosterol/total cholesterol ratio was significantly higher than that of controls and one of the indices of cholesterol absorption, campesterol/total cholesterol ratio, was lowered. One-dimensional statistical analysis revealed lowered contents of myristic (14:0), linoleic (18:2n6) and α-linolenic (18:3n-3) acid, whereas the content of oleic acid (18:1n-9) was higher in all lipid classes. In the all lipid classes, these changes were pronounced as higher content of monoenoic fatty acids and lower content of polyunsaturated fatty acids in both n-6 and n-3 series.

Fat Content in Low-energy Diet Fatty Acids and Metabolism of Cholesterol – Study VIII

Recently, a new attention has been paid to beneficial effects of high-fat diet on body weight reduction and metabolic profile in obese subjects. Reduction diets often cause low cholesterol levels, which can be caused by decrease in dietary cholesterol absorption and/or low cholesterol biosynthesis. The homeostasis of cholesterol under the condition of reduction diet is influenced also by the presence of noncholesterol sterols and fatty acids in the fat portion of the diet. In this study, we compared the effects of two hypocaloric diets with different proportion of fat on the homeostasis of cholesterol and fatty acid composition in blood and adipose tissue.

Forty-four obese subjects were submitted to 10 weeks´ low-calorie diet. Subjects were randomized into low-fat diet (LFD) (20-25 % of energy content) and high-fat diet groups (HFD) (40-45 %). Before and at the end of the intervention, samples of blood and subcutaneous adipose tissue were taken for subsequent analysis of fatty acid composition, plasma biochemistry parameters and noncholesterol sterols.

The diet-induced body weight and fat mass reduction were not different between the two diets. Plasma triacylglycerols were reduced during HFD only. Both diets reduced proportion of n-3 polyunsaturated fatty acids in adipose tissue and of saturated fatty acid in blood

triacylglycerols, with no difference between the diets. HFD induced a higher increase of monounsaturated fatty acids in blood triacylglycerols. No other diet-induced changes were found in proportion of major classes of fatty acids. In respect to individual fatty acids, the diets induced a number of changes in adipose tissue and blood, the changes, however, not being different between the diets. The HFD and LFD groups had lower cholesterol levels that were caused in HFD group by lower content of cholesterol in VLDL, whereas LFD group exhibited lower LDL cholesterol. We did not observe any changes in plasma lathosterol in both groups, but we detected in both groups lower concentrations of phytosterols campesterol and β-sitosterol.

5 DISCUSSION

Methodological studies

The analysis of fatty acid profile in plasma is necessary part of dietary studies[9]. The growing interest in some groups of fatty acids imposes high demand on their analysis, which has to be based on validated procedures in case of obtaining absolute data[10]. For example, PUFA n-3 are dietary supplements with proven hypocholesterolemic effect. The analysis of fatty acids by gas chromatography is nowadays a highly sophisticated procedure with vast number of detailed applications[11]. The favourite approach is the split/splitless injection mode of isolated mixture of fatty acid methyl esters into capillary columns that are in present produced in wide range of length as well as of polarity of the stationary phases so that the analysis could be easily set according to the requirements of the analysed profile of fatty acids. If the injection mode is set as a split/splitless one, the effect of injection parameters, like the split flow, the temperature of the split chamber and the injector configuration are of minor importance for fatty acid methyl ester analysis[12]. Therefore, our methodological studies focused on the effect of other steps of the analysis in lipid classes of human plasma.

In study I, we drew our attention to the column and integration software used. The identification of minor components in complicated chromatographic record need reliable configuration of the method and well-equipped integrating software. The difference between the content of major fatty acids and the fatty acids around the detection limit reaches three orders of magnitude. Even though, these minor fatty acids undoubtedly have clinical significance, e.g. conjugated linoleic acid, *trans* isomers of fatty acids, and Mead's acid (20:3n-9)[9,13]. The integration of minor peaks requires manual corrections; the modern software equipment with user-friendly interface allows for easy practice of these corrections. This is absolutely necessary for the separation of the peaks, for which it is not possible to set simple decision process suitable for the automatic handling of the data (e.g. unidentified component between 16:1n-9 and palmitoleic acid). In such a case, the imprecision would considerably influence the assessment of these two fatty acids. Moreover, palmitoleic acid is used a marker of lipogenesis[14].

In some studies, it is possible to decide which analytical matrix can be used. In study II, we focused on two analytical matrices (whole plasma and lipoprotein particles). The metabolism of fatty acid in plasma compartment is very complex and in tight relation to the metabolism of lipoproteins[15]. Furthermore, the release of fatty acids from adipose tissue and their binding to albumin play a role. The differences between the content of individual fatty acids in separate lipoprotein particles are negligible[16]. The little difference is probably caused

by wide exchange processes that include all lipid classes and involve all the lipoproteins. The variation of fatty acid content of LDL and whole plasma (study II) did not reach the values of the deviation of clinical set, for the LDL particles transfer most plasma cholesteryl esters and phospholipids[17]. Plasma triacylglycerols are transported predominately in VLDL and LDL particles. The comparison of fatty acid content in triacylglycerols in VLDL and LDL (study II) revealed considerable differences that even exceeded the total error of the analysis in case of PUFA.

Interestingly, clinical studies sometimes do not distinguish between the phospholipids and phosphatidylcholines. Phosphatidylcholines make for the most of phospholipids in human plasma and this is usually not further expanded on, it is only supposed that the content of fatty acid would be the same. This presumption was put into question in study III, and it was shown to be true. Yet, in some study design the carefulness is recommended – the observation of the metabolism of individual fatty acids in the lipid classes of plasma or the issue of positional isomers in lipid classes.

The analysis of minor plasma sterols is nowadays only a matter of marginal concern. The precursors of cholesterols are determined (above all) for the diagnosis on inborn errors of cholesterol biosynthesis and cerebrotendinous xanthomatosis, the analysis of phytosterols is indicated in suspected sitosterolemia[18]. Recently, the attempts for evaluation of liver function[19] and prediction of hypocholesterolemic treatment[20] based on the analysis of noncholesterol sterols appeared. Phytosterols and their saturated analogues, phytostanols, are suitable dietary supplements for cholesterol reduction with concomitant lowering of absorption of lipophilic vitamins. The maximum hypocholesterolemic effect of phytosterols (phytostanols) is reached in the daily dose around 2 g, higher intake has no additive effect and it is not recommended[21]. The monitoring of these sterols would be of clinical importance. Methodological part of the analysis of noncholesterol sterols is described in study IV. This study dealt with the effect of the type on injection technique and consequent derivatization on the calibration dependencies. The split injection mode brings about some disadvantage, but this was partly eliminated with the acetylation of the samples. It is also shown that the simultaneous determination of cholesterol and minor sterols in one chromatographic run is not possible; the contribution of individual steps of the analysis to the total error is studied in study IV, too. On the contrary to the determination of fatty acids, the gas chromatography burdens the total error substantially. Nevertheless, considering this error in comparison with the variance of the clinical set, the configuration of the method for the analysis of noncholesterol sterols is still recommendable.

Experimental study

Some authors associate the metabolism of cholesterol with the function of nervous system[22]; low cholesterol concentration was linked with the changes in mood and anxiety disorders[23]. Even though undesirable side effects of the hypolipidemic drugs (with annual turn-over about 20 billion dollars, ref. [24]) are closely watched, the drugs with cholesterol lowering effects could have some unpredictable effect on the central nervous system, which can be triggered outside the brain. Fenofibrate, the drug that does not primarily affect the cholesterol metabolism, had no effect on the concentration of the brain lipids. The molecule of (phyto)sterol is probably transferred through the blood brain barrier, because in phytosterolemic patients (suffering from accumulation of phytosterols), phytosterols had been accumulated also in the brain[25]. The changes of cholesterol content in the membranes of neuronal tissue were in study V connected with the changes in the sphingolipid content, which could reflect the effort of the cell to maintain the membrane fluidity, the important factor for the proper function of the membrane.

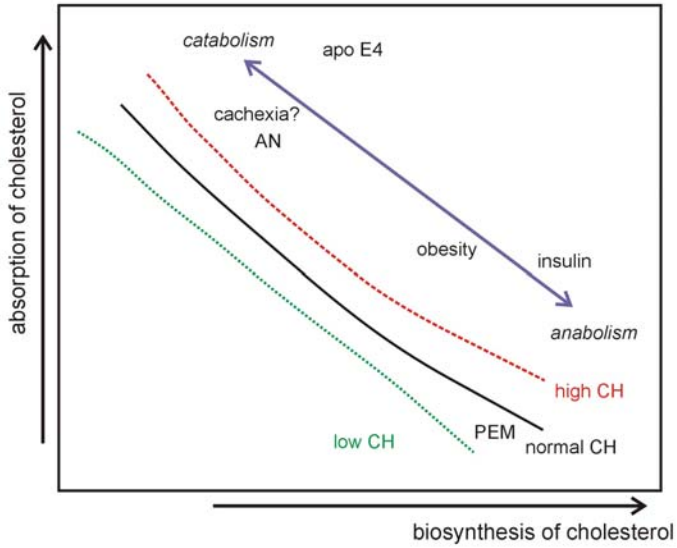
On the contrary to (chole)sterol molecules, the transfer of essential fatty acids (mainly docosahexaenoic and arachidonic) through the blood-brain barrier is obvious, because these fatty acids are absolutely necessary for the proper function as well as the development of the brain[26] and mammals are not able to synthesise them *in situ*. In Study V, both statins used and fenofibrate were proven to have an effect on the content of fatty acids in brain lipids. For example, in brain phosphatidylethanolamines, the content of stearic acid was lowered and the content of docosahexaenoic acid was higher after the administration of fenofibrate. The link between the metabolism of fatty acids in plasma and brain is probably more complicated than that of cholesterol, since both fenofibrate and the statins used influenced the content of several fatty acids in brain lipid classes only to some extent.

Clinical studies

The homeostasis of cholesterol is governed by several processes, which include the absorption in the gut, the biosynthesis (in liver and adipose tissue), and the conversion of cholesterol to bile acids; the last pathway represents the main route of elimination of the cholesterol from the organism. The adjustment of regulation processes and their sensitivity is also the result of cholesterol content in liver[27]. In our studies (Study VI, VII, VIII), we focused on the quantitative assessment of absorptional and biosynthetic processes. This evaluation can be accomplished by the determination of serum/plasma noncholesterol sterols.

Usually, there is a negative correlation between the markers of absorption (phytosterols) and cholesterol biosynthesis (the precursors)[28]. If the correlation is not observed, another factor plays a role, e.g. the release of (phyto)sterols from the adipose tissue during the dietary intervention (Study VIII); consequently, the cholesterol homeostasis is not established. The effect of several factors on the relationship between absorption and biosynthesis of cholesterol is indicated on the Figure 1.

Figure 1 Factors influencing the cholesterol homeostasis



CH – cholesterol, AN – anorexia nervosa, PEM – protein-energetic malnutrition

The changes in the metabolism of cholesterol and fatty acids in anorexia nervosa lead to (in some cases) hypercholesterolemia[29] with the PUFA n-6 deficiency[30]. Although in these cases the equilibrium between the cholesterol biosynthesis and absorption can be established, the high cholesterol levels are probably caused by the adjustment of the resorption process onto elevated level (Study VI). The patients had no changes in markers of cholesterol biosynthesis; anorectic states with the body weight far below the ideal value are supposed to use energy for absorption rather than for the cholesterol biosynthesis that require more energy. The high concentration of triacylglycerols in serum together with the observed hypercholesterolemia indicates that these patients have reduced catabolism of lipoproteins (Study VI).

The group of patients with protein-energetic malnutrition had lower BMI (Study VII) concomitant with enhanced level of cholesterol biosynthesis and diminished level of

exogenous cholesterol absorption. In plasma, the concentration of total cholesterol was probably decreased due to low HDL and LDL cholesterol. Cholesterol is intensively utilised for the cell membrane repair, the reverse cholesterol transport is limited together with the remodelation of HDL particles. The lower ratio of apoA-I/HDL-C (0.92 ± 0.31 vs. 1.34 ± 0.28 in control group; $p < 0.0001$ with Mann-Whitney test) indicates the replacement of apo A-I probably by serum amyloid protein A during the inflammatory response[31].

The opposite nutritional state, the obese (Study VIII), had in comparison with the control group higher value of lathosterol/total cholesterol ratio (1.50 ± 0.40 vs. 1.06 ± 0.05 in control group; $p < 0.01$ by Mann-Whitney test) and lower value of campesterol/total cholesterol ratio (1.68 ± 0.62 vs. 3.38 ± 0.31 in control group; $p < 0.001$ by Mann-Whitney test). High body weight is usually connected with increased biosynthesis of cholesterol both in non-diabetic[32] and diabetic with insulin independent type diabetes mellitus[33]. Insulin dependent diabetes is predominantly linked with higher absorption and low biosynthesis of cholesterol[33]; the anabolic effect of insulin probably plays a role. The factors influencing the adjustment of cholesterol homeostasis have also genetic background in case of absorption[34], but not in case of cholesterol biosynthesis[28]. The dietary intake of cholesterol and saturated fatty acids influences (with respect to the proportion of cholesterol absorption) the homeostasis of cholesterol[35]. When the body weight is slightly lowered without change in plasma insulin concentration, there is any change in cholesterol biosynthesis, whereas the metabolism of phytosterols is modified either in the sense of lowered absorption and/or deteriorated mobilisation of phytosterols from adipose tissue (Study VIII). With the respect to the fact that the obese prevailingly belong to the hypoabsorbers of (chole)sterol and the dietary intake of cholesterol as well as saturated fatty acids was not changed, the decreased concentration of phytosterols could be ascribed to the processes taking place in the adipose tissue (phytosterol accumulation in adipocytes or worsened mobilization of sterol molecules from adipocytes).

Malnutrition states and obesity bring about complex changes in fatty acid content of all lipid classes. The content of fatty acid is the product of many factors, which could be quantitatively assessed only with the highest diligence and many methodological difficulties. In clinical studies, it is possible to estimate some factors indirectly. From the results presented in Study II, it could be inferred that the fatty acid content in whole plasma compartment and LDL is roughly the same. Desaturation, elongation and β-oxidation processes could be estimated from the product/substrate ratios in relevant metabolic pathways (e.g. Study VI), the level of peroxidation of fatty acids by the determination of e.g. kinetics of conjugated dienes

in LDL or the total antioxidant status or the analysis of lipid peroxides. The process of the release of fatty acids from the adipose tissue is approximately proportional to the concentration of non-esterified fatty acid in plasma; the dietary intake of fatty acid could be evaluated by dietary records related to high-quality databases (e.g. Study VIII).

The relationship of fatty acid and cholesterol in plasma is projected to several levels of metabolism - directly into the formation of cholesteryl esters, indirectly by the formation as well as remodelling of lipoprotein particles, the influence on gene expression and interactions in cell membranes. Both protein-energetic malnutrition and anorexia nervosa are nutritional and metabolic disorders connected with essential fatty acid deficiency[30]. In the both groups, we observed lower content of n-6 polyunsaturated fatty acids caused by lower content of linoleic acid (Study VI, VII). The decrease observed for protein-energetic malnutrition in all lipid classes could be inscribed to enhanced lipid peroxidation and to the raised utilisation of cholesterol from lipoprotein particles in peripheral tissues. The protein-energetic group had also reduced molar percentage of n-3 polyunsaturated fatty acids (α-linolenic and docosahexaenoic). Since we did not observe enhanced catabolism of lipoprotein particles in anorexia nervosa, but rather retardation of the catabolism, the lowered content of linoleic acid in anorectic patients is caused by the deficiency of essential fatty acids rather than by sustained oxidative stress.

The obese group investigated had also lower content of n-6 polyunsaturated fatty acids (linoleic acid); whereas the concentration of triacylglycerols and total cholesterol (therefore also cholesteryl esters) were higher in the obese (Study VIII), the absolute concentrations of n-6 polyunsaturated fatty acids are higher than those of anorectic and protein-energetic malnutrition groups. This lower content has obviously significance. The insulin resistance that usually accompanies the obesity diminishes the activity of lipoprotein lipase in adipose tissue (particularly in visceral adipose tissue that is derived from the brown fat). Consequently, the secretion of triacylglycerol-rich VLDL particles is enhanced, HDL and LDL are richer in triacylglycerols; therefore, they are better substrates for hepatic lipase. The lipase forms small dense particles from LDL and reduces the number of HDL particles[36]. Hepatic lipase hydrolyses the *sn*-1 position in phospholipids (where there are monounsaturated and saturated fatty acids). During mild dietary restriction, linoleic acid is β-oxidised similarly to other polyunsaturated fatty acids[37] and no changes were observed in the contents of this fatty acid (Study VIII). In this study, we did not notice any change in the content of linoleic acid in plasma non-esterified fatty acids.

The higher content of palmitoleic acid (16:1n-7) reflect higher level of lipogenesis, whereas high concentration of non-esterified fatty acids indicate sustained release of fatty acid from adipose tissue. In the anorexia nervosa group (Study VI), both these two parameters were increased. On the contrary, in the patients with protein-energetic malnutrition (Study VII), neither the content of palmitoleic acid, nor the concentration of plasma non-esterified fatty acids were not changed. The anorectic patients have different distribution of adipose tissue compared to controls and realimentation brings about proportionally higher increase in visceral adipose tissue (in contrast to subcutaneous adipose tissue), so in the long run, the visceral fat has higher weight than in control groups[38].

The concentration of non-esterified fatty acids are higher in the obese, the low-calorie diet reduces them to the control values (Study VIII). The content of palmitoleic acid in plasma lipid classes and triacylglycerols of adipose tissue was decreased after the diet, which could point at the lowered lipogenesis. Concomitantly, the content of oleic acid was raised in the adipose tissue. Oleic acid is synthesised from stearic acid by delta 9 desaturase, which is induced by high cholesterol levels. During the diet, the sterols can accumulate in the adipocyte (Study VIII), therefore the desaturase activity can be increased. However, we did not observe the change in oleic acid content in plasma non-esterified fatty acids, but this could result from relatively slower mobilisation of oleic acid from the human adipocyte[39].

6 CONCLUSION

The methodological part of the dissertation focused on some aspects of the gas chromatography analyses of fatty acids and minor plasma sterols. The analysis of fatty acids were thoroughly worked on with the respect to the type of matrix analysed (lipoproteins of the plasma, phosphatidylcholines vs. phospholipids) and we determined the limits of the methods for the determination of some fatty acids and noncholesterol sterols. On the basis of the results obtained, the analytical processes were modified to meet the requirements of clinical studies.

The pathophysiological mechanisms responsible for the changes of metabolism of fatty acids and cholesterol in malnutrition and obesity are different. In anorexia nervosa, increased absorption of cholesterol together with slow catabolism of lipoproteins rich in triacylglycerols plays a role. Protein-energetic malnutrition is characterised by the diminished plasma content of cholesterol and essential fatty acid caused by their enhanced utilisation in peripheral tissues. Another factor important for the homeostasis of cholesterol and fatty acids is adipose tissue. This factor is notable during the low-calorie diet. Furthermore, we proved that the changes of plasma cholesterol can indirectly influence the metabolism of cholesterol and fatty acids in brain tissue in the experimental model.

7 SUMMARY

At present time, there is growing interest in two classes of lipids – sterols and fatty acids, which posses many different roles in living organisms. The concentrations of cholesterol biosynthetic precursors (lathosterol, desmosterol) reflect level of cholesterol biosynthesis, whereas the plasma phytosterol (campesterol, β-sitosterol) concentrations can be used for estimation of fractional absorption of dietary cholesterol. These groups are sometimes called together as noncholesterol sterols. The dissertation thesis dealt with the significance of analysis of noncholesterol sterols and fatty acid profile in various pathophysiological states.

The methodology part of the thesis covered some aspects of analysis of these compounds with capillary gas chromatography. We found that for the minor fatty acid analysis, both the type of the column and the software used play an important role. The gas chromatography step contributes by the least part to the total error of the procedure, whereas the effect of other steps (extraction, thin-layer chromatography and methylation process) is more pronounced. The comparison of fatty acid profiles in lipoprotein particles with low and very low density revealed differences, but these differences were highly interindividual. We also found that it is possible to replace the analysis of the fatty acid profile in plasma phosphatidylcholines with the less laborious analysis of the sum of phospholipids.

The sterol analysis was optimized by preferring the acetylation of samples and split mode of injection. The one-run analysis for the quantitation of minor sterols together with cholesterol was proven to be not reliable, therefore it was decided to use the method of two internal standards and two chromatographic runs. Comparison of the contribution of individual steps of sterol analysis showed that quantitative analysis of lanosterol and 5α-cholestanol is burdened with the error excluding these analytes from clinical evaluation.

In the experimental part of the thesis, the administration of hypolipidemic drugs (three statins and one fibrate) to brain lipids of male Wistar rats was studied. The statins used caused lowered content of cholesterol and diphosphatidylglycerol, which was counterbalanced by higher concentration of sphingolipids in brain lipids. The profiles of fatty acids were not considerably changed.

Clinical part of the thesis focused on the metabolism of fatty acids and cholesterol in anorexia nervosa, proteino-energetic malnutrition and reduction diet in the obese. The patients with anorexia nervosa had elevated concentrations of total cholesterol, triacylglycerols, HDL-cholesterol, campesterol and β-sitosterol. We also observed lower content of n-6

polyunsaturated fatty acids in all lipid classes. These changes seem to be the result of complex mechanisms including diminished catabolism of lipoprotein particles rich in triacylglycerols, unchanged level of biosynthesis of cholesterol and enhanced resorption of exogenous cholesterol.

Patients with proteino-energetic malnutrition exhibited lowered concentration of total cholesterol in plasma as well as in lipoprotein classes; concentration of apoA-I was higher, too. The concentration of lathosterol, indicator of cholesterol biosynthesis was not changed. The analysis of fatty acid profile showed higher content of monounsaturated and lowered content of polyunsaturated fatty acids. These metabolic changes in proteino-energetic malnutrition are connected more or less with higher utilization of cholesterol from lipoprotein particles in peripheral tissues.

Reduction diet set at the content of fat in the diet within the range of 20 and 45 energy % lowers body weight in order of percents, but it has no marked impact on cholesterol biosynthesis. The results suggest the diet-induced changes in fatty acid composition are controlled by the calorie deficit of the diet and the proportion of dietary fat plays a minor role. Ten-week low energy reduction diet induced changes in the metabolism of both cholesterol and phytosterols, which can be ascribed either to lowered dietary absorption or deteriorated turnover of phytosterols from the adipose tissue.

8 REFERENCES

1. Olson R.E.: *J Nutr* **1998**, *128*, 439S
2. Schumaker V.N., Puppione D.L.: *Methods Enzymol* **1986**, *128*, 155
3. Folch J., Lees M., Sloane-Stanley C.M.: *J Biol Chem* **1957**, *226*, 497
4. Tvrzická E., Mareš M., Votruba M., Hrabák P.: *J Chromatogr B* **1990**, *530*, 424
5. American Psychiatric Association v knize: *Diagnostic and Statistical Manual of Mental Disordes, 4th Edn.,* 5 American Psychiatric Association, Washington **1994**
6. Barendregt K., Soeters P.B. v knize*: Basics in Clinical Nutrition* (Sobotka L. Ed.), Galén, Praha **1999**
7. Wong D.T., Knaus W.A.: *Can J Anaesth* **1991**, *38*, 374
8. Anděl J. v knize: *Matematická statistika.* SNTL, Praha **1978**, str. 98-114
9. Seppänen-Laakso T., Laakso I., Hiltunen R.: *Anal Chim Acta* **2002**, *465*, 39
10. Schreiner M.: *J Chromatogr A* **2005**, *1095*, 126
11. James A.T., Martin A.J.P.: *Biochem J* **1956**, *63*, 144
12. Ulberth F., Schrammel F.: *J Chromatogr A* **1995**, *704*, 455
13. Holman R.T.: *J Nutr* **1998**, *128*, 427S
14. Aarsland A., Wolfe R.R.: *J Lipid Res* **1998**, *39*, 1280
15. Zhou L., Nilsson Å.: *J Lip Res* **2001**, *42*, 1521
16. Goodman D.S., Shiratori T.: *J Lip Res* **1964**, *5*, 307
17. Pownall H.J., Gotto A.M. Jr v knize*: Lipoproteins in health and disease* (Betteridge D.J., Illingworth D.R., Shepherd J Eds.), Arnold, London **1999**, str. 1-87
18. Kuksis A.: *J Chromatogr A* **2001**, *935*, 203
19. Nikkilä K., Miettinen T.A., Höckerstedt K.V.A., Isoniemi H.: *Transplant International* **2005**, *18*, 221
20. Hoenig M.R., Rolfe B.E., Campbell J.H.: *Atherosclerosis* **2006**, *184*, 247
21. Katan M.B., Grundy S.M., Jones P., Law M., Miettinen T., et al.: *Mayo Clin Proc* **2003**, *78*, 965
22. Dietschy J.M., Turley S.D.: *J Lipid Res* **2004**, *45*, 1375
23. Papakostas G.I., Ongur D., Iosifescu D.V., Mischoulon D., Fava M.: *Eur Neuropsychopharmacol* **2004**, *14*, 135
24. Maggon K.: *Drug Discov Today* **2005**, *10*, 739
25. Salen G., Horak I., Rothkopf M., Cohen J.L., Speck J., et al.: *J Lipid Res* **1985**, *26*, 1126
26. Qi K., Hall M., Deckelbaum R.J.: *Curr Opin Clin Nutr Metabol Care* **2002**, *5*, 133
27. Beynen K.S., Mostad I.L., Thoresen L.: *Am J Clin Nutr* **1980,** 33, 81
28. Kempen H.J., de Knijff P., Boomsma D.I., van der Voort H.A., Gevers Leuven J.A., et al.: *Metab Clin Exper* **1991**, *40*, 604
29. Gotto A.M. Jr, Pownall H.J. (Eds.) v knize: *Manual of Lipid Disorders. 2nd ed.* Williams & Wilkins, Baltimore **1999**
30. Holman R.T., Adams C.E., Nelson R.A., Grater S.J., Jaskiewicz J.A., et al.: *J Nutr* **1995**, *125*, 901
31. Banka C.L., Yuan T., de Beer M.C., Kindy M., Curtiss L.K., et al.: *J Lipid Res* **1995**, *36*, 1058
32. Stranberg T.E., Salomaa V., Vanhanen H., Mietinen T.A.: *Diabetes* **1996**, *45*, 755
33. Miettinen T.A., Gylling H., Tuominen J., Simonen P., Koivisto V.: *Diabetes Care* **2004**, *27*, 53
34. Berge K.E., von Bergmann K., Lütjohann D., Guerra R., Grundy S.M., et al.: *J Lip Res* **2002**, *43*, 486
35. Berglund L., Hyson D.: *Arterioscler Thromb Vasc Biol* **2003**, *23*, 1314
36. Lean M.E.J. v knize*: Lipoproteins in health and disease.* (Betteridge D.J., Illingworth D.R., Shepherd J. Eds.), Arnold, London **1999**, str. 881-895
37. Cunnane S.C., Ross R., Bannister J.L., Jenkins D.J.A.: *Am J Clin Nutr* **2001**, *73*, 709
38. Mayer L., Walsh T., Pierson R.N., Heymsfield S.B., Gallagher D., et al.: *Am J Clin Nutr* **2005**, *81*, 1286
39. Raclot T., Langin D., Lafontan M., Groscolas R.: *Biochem J* **1997**, *324*, 911

9 LIST OF THE PUBLICATIONS

1. Suljkovicová H., Viguerie N., Kunešová M., Millet L., Avizou S., Hejnová J., Hainer V., Barbe P., **Vecka M**., Tvrzická E., Langin D., Štich V.: Vliv výživy na metabolismus tukové tkáně. *Česk fysiol* **2001,** *50*, 57
2. **Vecka M**., Kunešová M., Staňková B., Žák A., Viguerie N., Hejnová J., Hainer V., Barbe P., Langin D., Štich V., Tvrzická E.: Změny složení mastných kyselin v séru a tukové tkáni u obézních žen po velmi přísné nízkoenergetické dietě. *Klin bioch metabol* **2001**, *9*, 119
3. Tvrzická E., **Vecka M.**, Staňková B., Žák A.: Analysis of fatty acids in plasma lipoproteins by gas chromatography-flame ionization detection - Quantitative aspects. *Anal Chim Acta* **2002**, *465,* 337
4. Zeman M., Žák A., **Vecka M.**, Tvrzická E., Romaniv S., Konárková M.: Treatment of hypertriglyceridemia with fenofibrate, fatty acid composition of plasma and LDL, and their relations to parameters of lipoperoxidation of LDL. *Ann NY Acad Sci* **2002**, *967*, 336
5. Žák A., Tvrzická E., **Vecka M.**, Romaniv S., Zeman M., Konárková M.: Effects of selected anthropometric parameters on plasma lipoproteins, fatty acid composition, and lipoperoxidation. *Ann NY Acad Sci* **2002**, *967*, 522
6. **Vecka M.**, Tvrzická E., Staňková B., Žák A.: Effect of column and software on gas chromatographic determination of fatty acids. *J Chromatogr B* **2002**, *770*, 91
7. Žák A., **Vecka M.**, Tvrzická E., Hrubý M., Novák F., Papežová H., Konárková M., Lubanda H., Veselá L., Staňková B.: Klinická a biochemická charakteristika mentální anorexie. *Čas Lék čes* **2003**, *142*, 280
8. Dlouhý P., Tvrzická E., Staňková B., **Vecka M.**, Žák A., Straka Z., Fanta J., Pachl J., Kubišová D., Rambousková J., Bílková D., Anděl M.: Higher content of 18:1 trans fatty acids in subcutaneous fat of persons with coronarographically documented atherosclerosis of the coronary arteries. *Ann Nutr Metab* **2003**, *47*, 302
9. Zeman M., Žák A., **Vecka M.,** Romaniv S.: Long-term hypolipidemic treatment of mixed hyperlipidemia with a combination of statins and fibrates. *Čas Lék Čes* **2003**, *142*, 500
10. Růžičková J., Rossmeisl M., Pražák T., Flachs P., Šponarová J., **Vecka M.**, Tvrzická E., Bryhn M., Kopecký J.: Omega-3 PUFA of Marine Origin Limit Diet-Induced Obesity in Mice by Reducing Cellularity of Adipose Tissue. *Lipids* **2004**, *39*, 1177
11. **Vecka M.**, Tvrzická E., Staňková B., Novák F., Nováková O., Žák A.: Hypolipidemic drugs can change the composition of rat brain lipids. *Tohoku J Exp Med* **2004**, *204*, 299
12. Zeman M., Žák A., **Vecka M.**, Tvrzická E.: Insulin resistance, metabolic syndrome and cardiovascular diseases. *Čas Lék Česk* **2005**, *144 Suppl 1*, 19-20, 22
13. *Žák A., Tvrzická E., Zeman M., **Vecka M**.: Pathophysiology of and clinical significance of polyunsaturated fatty acids n-3 family.* Čas Lék Česk ***2005****,* 144 Suppl 1*, 6*
14. Žák A., **Vecka M.**, Tvrzická E., Hrubý M., Novák F., Papežová H., Lubanda H., Veselá B., Staňková B.: Composition of plasma fatty acids and non-cholesterol sterols in anorexia nervosa. *Phys Res* **2005**, *54*, 443
15. ***Vecka M.,*** *Žák A, Tvrzická E.: Sterols, Especially Cholesterol and Phytosterols, in Human Metabolism. Kapitola 6.54.3.7. in* Physiology and Maintenance, from Encyclopedia of Life Support Systems (EOLSS), *Developed under the Auspices of the UNESCO, Eolss Publishers, Oxford, [http://www.eolss.net] – kapitola v online encyklopedii*
16. Zeman M., Žák A., **Vecka M.,** Tvrzická E., Písaříková A., Staňková B.: N-3 fatty acid supplementation decreases plasma homocysteine in diabetic dyslipidemia treated with statin-fibrate combination. *J Nutr Biochem* **2006**, *17*, 379
17. **Vecka M.**, Richterová B., Žák A., Tvrzická E., Šrámková P., Staňková B., Klimčáková E., Štich V.: Změny složení mastných kyselin v séru a tukové tkáni v závislosti na obsahu tuků v nízkoenergetické dietě. *Čas Lék Česk* **2006**, *145*, 464
18. Tvrzická E., Staňková B., **Vecka M.,** Žák A.: Potravinové doplňky s rybími oleji – složení mastných

kyselin. *Čes.-slov. Pediatr.* **2006**, 61, 513

19. *Tvrzická E., Žák A.,* ***Vecka M.,*** *Staňková B.: Fatty Acids in Human Metabolism. Kapitola 6.54.3.5 in* Physiology and Maintenance, from Encyclopedia of Life Support Systems (EOLSS)*, Developed under the Auspices of the UNESCO, Eolss Publishers, Oxford, [http://www.eolss.net] – kapitola v online encyklopedii*
20. Žák A., Zeman M., **Vecka M.,** Tvrzická E.: *Kyselina nikotinová – lék neprávem opomíjený.* Čas Lék Česk ***2006,*** 145*, 825*
21. **Vecka M.,** Žák A., Tvrzická E.: Rostlinné steroly jako funkční potraviny. *Čas Lék Česk* **2007**, 146, v tisku